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RELATIONSHIP OF STEROIDS AND PROTEINS IN THE ANIMAL ORGANISMS

by

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(Speech given at Lyon 25 April 1955)

In 1951 in a report to the 28th French Medical Congress (17), we insisted on the importance of the study of lipoprotein relation between blood and tissues in the biochemistry of steroids.

The subject doesn't seem to have reappeared in literature since then and I thank Prof. Enselme for remembering "this subject which is dear to me".

I will attempt to answer his invitation and that of the Society of Biological Chemistry by combining a critical review of the question with our thoughts and first reuslts concerning the relationship of steroids and proteins, principally in the human organisms.

The relatipnship between proteins and lipids preoccupy the biochemist at all times, not only because its structure, role and the mechanism of their synthesis escape them, but also for a less direct reason: these relationships, often unsuspected, prevent the complete extraction of the lipids; they constitute a dangerous obstacle to the knowledge of lipids - and of the steroids in particular - in the biological products.

The power of lipoproteins to emulsify and dissolve independant lipids is added to these difficulties: this type of "solution" can, in turn, also resist to extraction.

Finally, we must bear in mind that, as pointed out by Enselme (24),

"any protein whatsoever, when subjected to a more or less advanced stage of denaturation" "may fix normally independent lipids" and subtract them from the action of usual solvents.

One may therefore logically state (17) that the chemical study of steroids, in the tissues and in blood, must be partly considered in light of protein chemistry.

The first idea on lipoproteic complexes dates back to 1866, when Hoppe-Seyler noted that tissues had to be treated with a protein denaturation so as to extract all lipids by ether (44).

In 1918, Bang (1) maintained for the first time that the blood serum cholesterol is connected with the proteins and in particular with the globulins. In 1926, Troensegaard and Kondhal (72) concluded - on admittedly, rather fragile foundation - that cholesterol is a prosthetic grouping of serumglobulin.

In our opinion, these are the most obvious steps in the history of lipoproteins up to the day when Machebœnf began the first methodical work on these substances. (55, 56).

The lipoproteins were originally essentially considered as "synapses", vague lesions, between proteins and lipids <u>where the properties of both constituents are modified or masqued</u> (Macheboeuf).

This definition must be broadened at present: the word lipoprotein means any lesion, protein-lipid, in which at least the lipidic constituents are not directly detectable by their reactive or by their specific solvents. This definition does not prejudge anything, neither the type of relation, nor the complex concentration into lipids, nor the nature of the bound lipids. It therefore also holds true for the relation between steroids and proteins.

However, it is imperative to be able to distinguish these particular lipoproteins and for lack of a better expression shall use steroidoproteins. By this term we mean all lipoproteins with a lipidic fraction wholely or partly constituted by steroids.

Our knowledge of steroidoproteins is mainly based on chance finds, made during general lipoprotein tests. Even the most thoroughly studied lipoproteins hold many unknowns in this area. As these are the most notorious steroidoprotein sources, a reasonable inventory of the lipoproteins should be drawn.

The collected materials will help us investigate the relations between proteins and steroids.

We will end with a study of methods of investigation and by an exposition of some results.

I. Animal lipoproteins in general

A number of lipoproteins are known to us, mainly in the blood plasma. A number tend to turn it into a classic concept, and to tabulate its properties, and even the number of its substances. One must not forget that the study of these relations is at its very start, and that an unlimited number of lipoproteins can exist in theory. This is partly brought out in the following work.

1. Lipoalbumins

The question of relations between lipids and albumins remain rather obscure.

Macheboeuf, was the first to isolate a lipoprotein, which had an albumin

proteic faction, from blood plasma (horse) (55, 56). This author has shown recently (57, 58) that 18 p. 100 cholesterol esters (10,4 p. 100 total cholesterol) and 23 p. 100 phospholipids are fixed on one albumin of horse serum. This "acid synopsis" - as named by the author - has a molecular weight of approximately 85.000.

Gorter (36) confirmed Macheboeuf's observations as early as 1946: he isolated as non-precipitating lipoprotein from horse (or bovine) serum, approximately pH 7, by means of semi-saturation with ammonium sulfate with a molecular weight of approximately 70.000. This author found 13,1 p. 100 of total cholesterol and 39,6 p. 100 of total lipids; the curve of spread in monomolecular layer in function of the pH was practically identical with that of serum albumin. The solubility characters, the molecular weight and and curve of spread led Gorter to agree with Macheboeuf on the albumin nature of the complex protein.

It is known that certain small globules are not precipitated (by $(NH_4)_2SO_4$ more than the albumine in semi-saturation and in a neutral medium, this precipitation being also a function of the concentration and mainly of the pH. A simple operation evidently does not allow a precise knowledge of the protein nature. On the other hand the molecular weight and partly the curve of spread, seem to confirm the albuminous nature of the Macheboeuf complex.

The latter has also shown that the acid synopsis only shows one single component for electrophoresis, with phosphate strip pH 7,7, and that its mobility is quasi identical with that of serum albumin (57). The rate of sedimentation during ultracentrifugation is slower (S = 2,5^{-4}) than that of

albumin, but this may be explained by the high lipid contents. The possibility of denaturation during isolation of the acid synopsis was also studied by Macheboeuf (56, 57).

In short Macheboeuf's lipoalbumin or "acid synopsid" is one of the most studied lipoproteins. A strong probability, but no absolute certitude, exists in favor of the albuminous nature of this complex.

Other authors don't even mention this albuminous nature.

In fact we shall soon see that methods of fractioning (graining by alcohol according to Cohn, electrophoreses according to Tiselius) have repeatedly led to the isolation of fractions rather amalogous to acid synopsis but having certain characteristics leading one to believe that it is a question of small globules. Confusions certainly exist here as a result of (1) the imperfection and the non-equivalence of techniques; (2) the juxtaposition of results in connection with various animal types.

Whatever the case, it is now too early to affirm, as done by some, that Macheboeuf's acid synopsis is identical to a_1-lipoprotein of which we shall speak below.

In 1941, Blix, Tiselius and Svensson (6) isolated an albumin containing 1,1 p. 100 cholesterol and 2,2 p. 100 phospholipids from human serum by electrophoresis. This human lipoalbumin differs from Macheboeuf's equine or bovine but its study is less advanced and the possibility of a mixture exists.

The same holds true for the crystallized human serumalbumin complex and for fatty acids obtained by Kendall, in 1941 (48).

Let us add that several steroids of the cardiotonic aglycon group

mainly join serum albumins (25, 43).

It is certain that physiological, pathological and pharmacodynamic relationships exist between serum and lipid albumins. These relationships are little known and vary from one animal species to the next.

2. Lipoglobulins

The principal constituents of this group are for the present those usually called the a and B-blood plasma lipoproteins.

Blix, Tiselius and Stenson (6) in 1941, found a high percentage of lipids associated with alpha and beta electrophoric globulins from normal human serum. It has been recognized as a result of this work, that a and B-plasma or serum globulins contain lipoproteins named respectively a and B.

The name lipoglobulins seems preferable for use here as it allows a distinction between these relationships and the lipoalbumins.

A. The a lipoglobulins (or a lipoproteins)

Isolated in the raw state by electrophoresis (6), they were later isolated by salting out, by alcohol in the IV-I Cohn fraction (63).

The a lipoglobulins are the least known of the lipoglobulins. They are easily denatured and are nearly always contaminated by other proteins, notably by albumins.

"Cholesterol" presents interesting particulars in connection with a lipoglobulins. It may easily be extracted by leaving the protein in contact with ether. An unexpected fact, which we have been able to verify since Batchelor (3), is that denaturation by 60° heat makes it impossible to extract this cholesterol with ether. In these tests we used total normal human blood plasma alpha-globulin obtained through precipitation under the

form of Fraction IV-1 by Cohn's method 6. Even chloroform agitation prolonged for over 24 hours attained an approximate production of 30 p. 100 (in Lieberman colorimetric equivalents) as compared to the quantity extracted without previous denaturation. We wonder if this really concerns cholesterol (See Section 3).

In 1952 Lewis, Green and Page (52, 53) using ultracentrifugation by floating isolated two a-lipoglobulins which they respectively named a_1 and a_2 lipoprotein. These isolations were made more precise and partly confirmed by electrophoresis, but the authors noted large quantative variations dependant on the animal species.

The a_1-lipoglobulin (or a_1 lipoprotein of Lewis and others) is the main constituent of a-lipoglobulins. It precipitates with a_2-lipoblobulin in the Cohn faction IV-1. In the average in human plasma it represents approximately 40 p. 100 of the total a_1-globulins 10 p. 100 of which must still be identified The a_1-lipoglobulin is considered by Gurd (37) and his collaborators as a completely individualized molecule 300 Å long to 50 Å wide with a molecular weight of approximately 200000.

In 1950 Weimar, Mehl and Winzler (77)by salting out human serum, isolated an acid glucoprotein or mucoprotein containing aproximately 30 p. 100 glucides and approximately 3,6 p. 100 of total lipids. It is an interesting example of a mixed "gluco-lipoproteic" composite; it is a proof of the diversity of lipoproteic relationships. This mucoprotein has the same electrophoretic mobility as the a_1-serum lipoglobulins (See Table I).

The a_2-lipoglobulin (or Lewis and others lipoprotein a_2) is hardly known. It has a flotation speed between the B and the a_1-globules. It only represents

approximately 3 p. 100 of the total a_2-globules more than 90 p. 100 of which must still be identified (Table I).

B. The B lipoglobulins (or B lipoproteins)

B lipoglobulin (or B-lipoprotein or B_1-lipoprotein) with Macheboeuf's lipoalbumin, is the best known lipoprotein of blood plasma. It represents an average 40 p. 100 of the normal human blood plasma globules, but the number varies greatly from one individual to the next and according to age and sex (2, 52). It precipitates in the III-0 fraction of Cohn from which it has been isolated by ultracentrifugation (63). The B lipoglobulin contains dry weight 8 p. 100 free cholesterol, 39 p. 100 cholesterol esters, 29 p. 100 phospholipids and only 23 p. 100 polypeptide (Oncley (63)) approximately 75 p. 100 of the serum "cholesterol" is bound in this complex which represents up to 5 p. 100 of the total plasmatic proteins (Table I). Small quantities of carotenoids and oestriol have been identified in B lipoglobulin.

According to certain authors (37, 76) it is a question of a definite chemical individual, with a spherical molecule 185 Å in diameter and a molecular weight approximately 1,300,000. However certain recent immunochemical works (32) show that that in fact it is only a question of a mixture.

The great lack of stability of this matter makes its study extremely difficult. It is nearly instantly denatured by coagulation and desication. The lipidic fraction is rapidly oxydized by contact with air and this oxidation seems to be started by that of carotenoid traces and by that of non-saturated fatty acid esters. An appropriate anti-oxidant has not been

found to date.

Recent immuno-histochemical tests made by Coons (11, 12) method, have shown that the B lipoglobulin easily passes into the cell cytoplasm. This might be a basis for the belief that this lipoprotein is used by the lipids as a means of crossing through the cellular membrane.

C. Mc Farlane's Protein-X

Isolated in 1935 (59) by ultracentrifugation this protein appears to be identical with the B-lipoglobulin.

D. y lipoglobulins

Blix, Tiselius and Svensson (6) observed in 1941, that small quantities of cholesterol (0,4 p. 100) and of phospholipids (1 p. 100) are linked with normal human serum gamma-globulins (impure fractions).

Leinwand (50) has recently found electrophoretic disturbances with a high number of lipids in association with gamma-globulins. It is known that the major part of antibodies belong to this group the liberation of which from the lymphocytes on is controlled by oxygenated steroids in 11 of the suprarenal cortex (23).

Table I lists a few of the properties of the principal known blood plasma (mainly human) lipoproteins including their relations to the large electrophoretic fractions and their principal constituents.

3. Lipoproteins of unknown structure

Numerous works have appeared during these last few years on the isolation of blood or tissue lipoprotein fractions. Many of these do not provide sufficiently complete or precise data to be mentioned in detail here. They

TABLE I

Blood Plasma

(The Table refers to human plasma, except for the Macheboeuf lipoprotein. The lipoproteins are in thick letters, except for the gamma-globulin fractions).

Fractions	Identification degree	Principal identified constituents	Molecular weight	Isoelectric point	Cohn fraction	Total lipids p. 100	Total cholesterol	Other identified steroids
Albumin	90 p. 100 (?) variable heterogeneity	Serum albumin	70.000	approx. 5	V			
		Macheboeuf lipoalbumin (horse, beef)	70.000 at 85.000			40	13	
a_1-globulins	approx. 90 p. 100 are identified	a_1-mucoprotein (acid glucoprotein)	45.000	3		3,6		
		a_1-lipoglobulin	200.000	5,2	IV-I	35	12 (*)	17-cetoster.
a_2-globulins	approx. 10 p. 100 are identified	Ceruloplasmin	150,000	4,4	IV-I	?	?	
		a_2-lipoglobulin (C_1, C_2 complement)						
		Fetuin (newborn)	35.000	3,5				
		Haptoglobulin	120.000	4,9				
B-globulins	approx. 70 p. 100 are identified	Transferrin	90.000	5,8				
		B-lipoglobulin	1.300.000	5,4	III-0	77	31 (**)	oestriol (400 y p. 100)
		(prothrombin)	63.000	4,2	II + IIIV			
y-globulins	approx. 25 p. 100 are identified	Fibrinogen isohemoagglutinins, various antibodies, etc.	330.000	5	II + IIIV	1,4	0,4	

(*) According to (9)
(**) According to (63)

often show an imperfection of methods and the lack of equivalence in the latter causes confusion in these works.

A. Gofman's lipoproteins

These lipoproteins, isolated by ultracentrifugation of flotation of human and rabbit serum in 1949, show an analogy to B-lipoglobulins by their lipidic fraction but are distinguished from the latter by the speed of flotation. It could be a question of a B artifact caused by elevated ionic concentrations.

They contain approximately 30 p. 100 total cholesterol, vitamins and liposoluble hormones (Oestriol ?) and have the property of emulsifying or dissolving neutral fats.

B. Lipoproteic fractions by Goldwater, Lewis, etc.

The fractionings of plasmatic lipoproteins reached by an analogous technique by Goldwater and his collaborators (35), by Lewis and his collaborators (52, 53) etc. should be paralleled to Gofman's work.

C. Dandliker's erythrocyte lipoproteins

In 1950, Dandliker and his collaborators (14) isolated a lipoprotein from human erythrocytes, "elinine", which contains an R_h factor and A and B substances.

D. Folch's "proteolipids"

Isolated from the brain (26, 27, 28, 29, 30) these particular lipoproteins are characterized by insolubility in water and by strong solubility in chloroform and in chloroform and methanol mixtures. One of the fractions would contain 45 p. 100 lipids, mostly fatty acids and phospholipids.

The paradoxical solubilities of these bodies recall the solubility of certain serum amino acids in petrol ether as only caused by the presence of phospholipids (78).

II. Blood and tissue steroidoproteins

Most of the lipoproteins which have just been mentioned are, in varying degrees, steroidoproteins. The preceding enumeration leaves one with the impression that these present a rich and little known field.

In the following section, we propose to find out whether there are any other indications in favor of the existance of specifically steroidoproteic relations in the biochemistry of all tissues, and what would eventually be the physiological function and the chemical nature of these relations.

1. In the blood

We have seen that 3 types of protein related steroids have been identified in blood plasma; cholesterol (free and esterified), oestriol and 17-cetosteroids. The precise identity of the latter is not known.

During recent experiments which will be described in Section 3, we noted the existance of relationships between proteins and certain corticosteroids in the human blood plasma.

It should be noted that the steroids always seem to be accompanied by other lipids in the protein relations in which they have been identified so far.

Oestriol - B_1-globulin is the best known plasmatic steroidoprotein from physiological point of view. According to Szego and Roberts (68) oestriol is found in the form of glycuronide. The oestriol-glycuronide -

B_1-globulin would be synthesized in the liver and would represent the principal circulating type of the oestrogens in the organism.

In connection with this, Pincus (64) made an interesting contribution to the steroid hormones in a physiological way; steroids usually considered "hormones" (Testosterone, B oestradiol, hydrocortisone) only exercise their characteristic effect by the means of "catabolites". In many mammals, oestriol would be the active principal oestradiol catabolite. It is interesting to note that there are only approximately seven micrograms of oestriol in an entire liter of human blood, two-thirds being connected to proteins.

2. In extracts of steroid producing tissues

Works on tissular extracts give less information on the steroidoproteic relations than blood. Gitlin and his collaborators (33), in applying Coons' (11, 12) immuno-histochemical method found that albumin, B-lipoglobulin and y-globulin of blood plasma normally penetrate into the cellular cytoplasm. It would then seem probable that any tissular extracts, even when devoid of blood, contain more or less the same steroidoproteins as blood plasma.

However, similar relations in the tissues have not yet been definitely identified, even in the glandular steroid producing tissues. The only arguments for the tissues are based on supposition and probability.

A. In the testicle

The extraction of testosterone from bull and mainly from pig testicles gives very poor results if the methods of extraction used are not energetic enough, such as simple digestion (for a few days) with alcohol at ordinary temperature. The results are greatly improved if the extraction is made by

boiling.

A possible argument in favor of a testosterone-protein relation results.

B. In the placenta

Diczfalusy (20) has suspected the existance of a protein-progesterone relation in the human placenta because the tissue alkaline hydrolysis still liberates progesterone after 3 days, despite ether extractions twice a day. The argument remains problematic mainly becuase it is not constantly verified.

C. In the suprarenals

The sometimes large differences in the steroid production of suprarenal extracts, according to the method employed, seemed to stem more from the processes of extraction and hydrolysis than from variations between species, individuals or seasons. This is an argument of presumption similar to that of the testicle.

D. In the parathyroids

Raoul and his collaborators (65) have quite recently isolated a lipidic non-nitrogenous probably sterolic substance from parathyroids (pig, ox, horse), which seemed to contain the entire hypercalcificating activity of these glands. Isolation was only achieved after prolonged boiling of a proteic fraction in absolute ethanol.

3. Relations with apoenzymes (cofermentary role)

A study of the enzymatic systems in relation to steroid metabolism may be of interest.

The enzymes which take a direct part in the biogenesis or in the catabolism of steroids are little known and at present there is no indication of

a direct relation with these ferments. There exist presumptions in favor of the existance of such relations for other enzymatic systems connected with steroids:

Certain corticosteroids could be coferments of the alkaline phosphatase (74).

On the other hand, one may note the inhibition of the following enzymatic systems after suprarenalectomy: phosphorylase of muscle and liver (73), arginase of the liver (49), glucomutase of the muscle (10). C. F. Cori and his collaborators noted an in vitro antagonism between hexokinasis and the corticosteroids (70). Finally, Hayano, Dorfman and Yamada (39) noted the inhibition of d-aminoacidoxydase by desoxycorticosterone and concluded on the possibility of a reaction between the steroid and the apoenzyme of the oxydase.

Dirscherl (21, 22) has described a stimulating action of androgenes and oestrogenes steroids with respect to an enolase to a hexokinase and a carboxylase. Administration of oestrone in the ovarectomic rabbit restitutes the uterus actomyosine concentration and stimulates the adenosine triphosphatase activity. (13)

Progesterone stimulates the activity of uterus lactic dehydrogenase and inhibits that of the DPNH oxydase while the two systems would be stimulated by the natural oestrogens (4).

It is evident that these various stimulating or inhibiting actions could be indirect and in no way prove the existance of a relation with the ferment apoenzyme. One may however consider such a relation along this line, the relation of certain cortico-steroids with insulin apoenzyme is

another attractive hypothesis which seems to be supported by certain pathologic factors.

4. The peptidic relation of bilious salts

Some have considered the "peptidic" relation of bilious salts the indication of an origin by cleavage from a heteroprotein with bilious acid as a prosthetic group. Nothing is less certain. In fact, this is a case of a pseudo-peptidic relation analogous to that of hippuric acid. At present one may admit that the coenzyme A (CoA) catalyses the synthesis of such relations (8, 66). The fact is demonstrated for the synthesis of hippuric acid which can be schematized:

BzOH + CoApyrophosphate → BzCoA + pyrophosphate

BzCoA + glycocol → Bzglycocol + CoA

This holds true for the synthesis of other acylated derivants from various non-aromatic amino acids (47, 54, 69).

Let us add, in parenthesis, that the synthesis of the actual peptidic relation does not seem to be explained by the intervention of the CoA.

The bilious salts offer no argument in favor of a steroidoproteic relation.

5. Physiological role

The physiological role of the steroids or lipoproteic complexes has often been discussed. We have mentioned it above, in connection with the role of oestriol glycuronide - B_1globulin. On the other hand we have seen that immuno-histochemical works have shown the penetration of lipoproteins in the cellular cytoplasm. The ancient concept of a "transport" role seems

thus to be confirmed and made more precise. Finally, the role of these relations is without doubt as numerous as the actual relations.

6. Chemical or physical nature

The nature of the relation between protein and lipids evades us. The types of relations are certainly numerous and they certainly vary up to a point for a molecule during the metabolism. The phenomenons of solvation and of association certainly play a part in the latter - with all the differences that these terms contain in their definition of the relation between solvent and dissolved body. The simple play of Van der Waals' forces where the juncture by the hydrogen bridges intervene beside relations resisting to the most energetic analysis process.

At this writing, certain proof of the existance of steroidoproteins only exists for blood plasma. Here the cholesterol far outdoes all the other steroids by its semi ubiquity and by its nigh numbers. However, the study of blood plasma that of fermentative systems, and that of tissular extrarts give the impression that quite a few relations with steroids other than cholesterol escaped the researchers for various reasons.

III. The methods for the study of steroidoproteins. A few results

How to approach the study of these relations in practice? The method naturally varies, according to one's desire to isolate the holoprotein or only the prosthetic steroids. Here we will only be concerned with the second point of view.

The question brought up before an instant seems at first to deserve our whole attention: why does cholesterol, of the numerous steroids identified

in the animal tissues, seem to dominate on this point the other steroids in the steroidoproteinic relations?

Physical or technical problems? In other words: do the facts conform with the appearances? - and in this case, what is the predominence of cholesterol? - ; or does the so-called cholesterol hide other steroids? and where in this case is the technical lack?

For numerous reasons - their listing would carry us beyond the subject of this conference - cholesterol seems to be the steroid most frequent in the animal organism. The basic reason remains obscure. It could be suggested that cholesterol is a precurser of other steroids. However this theory becomes dubious and the fact, at least, certainly not exclusive, as proven by the wonderful works by the schools of Hechter, Zaffaroni and Pincus (40, 41, 45, 51, 64, 79) and those by Bloch (7), Haines (38), Vestling (75)... Cholesterone may be considered a certain reserve-plug slightly like hepatic glycogen?

Whatever the case we believe that "cholesterol", as usually found, might hide a great number of other steroids. These are the reasons:

Two great causes for error actually exist in the study of steroids contained in the protein rich areas (blood, tissular extracts):

1. Inadequate extraction methods
2. The current cholesterol (17) identification methods

First cause of error: extraction procedures

Let us remember that our object here is the quantitative extraction of steriods attached in any manner to proteins and not th at of the actual proteic complexes.

Nelson and his collaborators (50) while studying the dosage of the 17 blood hydroxycorticoids stated in 1951 that there is no indication of notable conjugations among these steroids. They stated that as a matter of fact, neither incubation with B-glycuronidase nor acid action at ordinary temperature increased production. These authors usually - and others have since (5, 60, 62, 71) - make the extraction either at the blood pH, or more rarely at pH 1.

These methods appear insufficient after what we have said about protein relations. The method of extraction by dialysis perfected by Zaffaroni (80), also disregards these relations.

A "tradition" has long implied that there are no relations with blood proteins, and that all attempt at hydrolysis should be feared. This is an absurd prejudice inspired by superficial or incomplete studies.

We have admitted that for obtaining a quantitative extraction of steroids of blood or of tissue, it was necessary a priori: (1) to try to break the hypothetical connection with the proteins; (2) to find out whether there are any non-proteic relations antagonistic to ordinary extractions.

The liberation of steroids from their proteinic connections may be attempted either by denaturation, or by the more or less complete destruction of the proteic molecule.

The denaturation with concomitant extraction such as hot extraction with alcohol-ether, with alcohol-acetone etc., give better results than those obtained by "careful" cold extractions by means of slightly denaturing solvents such as chloroform.

Here I will mention that certain denaturations sometimes lead to para-

doxical results: above, we have described that progressive heating, below 60° of a lipoglobulin hinders cholesterol extraction.

We think that the most certain method for the quantitative liberation of steroids in these mediums is the use of proteolytic enzymes, for example trypsin or papaine.

We have turned to this ferment in the work on human blood steroids recently begun in our laboratory.

The inconstancy of the activity of ordinary papaine is avoided by the choice of a very pure papaine. The greatest difficulties are: (1) in the choice of ferments after the fermentative action; (2) in the solubility of the ferment in most solvents; (3) in the purification of the extract; (4) in the resistance of certain proteins to the proteolytic ferments.

General proposed technique

Here are the main lines of the technique we momentarily adopted for the extraction and for fractioning.

1. Proteinase digestion

50 to 100 cm^3 of blood plasma (or of tissular extract) are divided into 3 or 4 equal portions. One or two portions are used as controls and are treated like the samples but without addition of papaine. 5, 10 and eventally 15 g of papaine per 100 cm^3 are respectively added to the other portions. It is allowed to be digested for from 24 to 48 hours at ordinary temperature or at 37°.

2. Extraction and acid hydrolysis

Each sample and each control are then subjected to extraction by a number of successive solvents of varied polarities and general properties.

TABLE II

Dipolary moments (in liquid phase) of a few solvents which are not soluble in water (*)

Polar solvents (at dipolar moments superior to 1,5 D)		Non-polar solvents (at dipolar momemts below 1,5 D)	
n-butanol	: 1,66	Benzene	: 0
Ethyl acetate	: 1,85	Carbon tetrachloride	: 0
Acetophenone	: 2,93	Diethylic ether	: 1,14
Nitrobenzene	: 3,95	Chloroform	: 1,15

The first stage of the phase of extraction and acid hydrolysis is usually conducted along the following plan:

Product of digestion (or control)

Extracted 4 times in 30 minutes with 50 p. 100 $CHCl_3$ (chain belt).

Chloroformic extracts (A)

I Watery phase (Non-proteic or papaine resistant (?) relations and relations non-soluble in $CHCl_3$). Extracted 4 times 30 minutes with 50 p. 100 n-butanol (chain belt)

Neutral butylic extracts (B)

II Watery stage Add 10 p. 100 concentrated HCl. Boil 10 minutes. Cool and extract 4 times 30 minutes + 50 p. 100 $CHCl_3$.

"Acid" chloroformic extracts (C)

III Watery phase Extract 2 times 30' + 50 p. 100 of butanol

"Acid" butylic extracts (D)

Waste water phase

(*) u is expressed in Debyes 1 D = 1.10^{-18} U.C.G.S.

The first stage therefore results in 4 extracts. The C and D extracts are rinsed with a minimum of water saturated with NaCl. The neutralized rinse waters are repeatedly re-extracted with $CHCl_3$ and these chloroformic extracts are respectively added to C and D. Following dehydration by NA_2SO_4 the four series of extracts are evaporated under reduced pressure to dryness either separately, or more frequently, in pairs A + B and C + D.

3. First fractioning

Every residue is taken up by ether devoid of peroxides. The etheral solutions are fractionated by successive extractions with $NaHCO_3$ at 10 p. 100 and then with NaOH 2-n. One has:

a. Products soluble in $NaHCO_3$. Reacidify and re-extract: "acid" fraction.

b. Products soluble in NaOH. Reacidify and re-extract: phenolic fraction.

c. Products insoluble in both these mediums (direct etheral phase and rinsing phases before acidifications): neutral fraction.

Every fraction is evaporated to dryness after dehydration.

4. Optional rough doses

In an aliquot part of the residue, one may administer doses grosso modo by the usual techniques, the 17 cetosteroids (in the neutral and in the phenolic fraction), the 17-hydroxy-corticosteroids (especially in the neutral fraction), the oestrogens (especially in the phenolic fraction), etc.

5. Second fractioning

The residues of each of the 3 acid, neuter, and phenolic fractions are treated by the Girard P reaction.

Then, (a) the ketonic fraction is divided into alpha-hydroxyled + non-hydroxyled and into beta-hydroxyled by digitonine (*). We pass to stage 6;

(b) The hydroxyled products (mainly cholesterol) are separated from the non-ketonic fraction by the action of $C_6H_5N.SO_3$. The hydrolysis of the formed sulfate esters is achieved with diluted H_2SO_4. One may then either fraction by digitonine or directly pass on to stage 6.

6. Chromatography

The 4 or 5 obtained fractions are separately chromatographed on silicate by Mg + 50 p. 100 of celite, then on Brockman alumina (II-III activity). Alumina chromatography is sufficient in most cases (systems of successive solvents: hexane-benzene-methanol). Paper chromatography is being studied.

7. Identification and dosage

Identification may only be achieved by the combination of a number of tests: absorption curve in the I-R and in the U-V and formation of specific composites. This last test, added to the arguments drawn from the preceding fractionings, is sufficient in certain cases for instance in that of cholesterol which we will describe at the end of the work.

The dosage, after identification, may be made by a color reaction or by gravimetry.

Results

The results of this work, which is still under study will be published in detail at a later date. We will later give the results of the application of this method to the specific dosage of cholesterol in blood plasma.

Given here are a few total results concerning two pools of 450 cm^3 of human blood plasma (including the controls) so as to illustrate the influence

(*) The criticism of the fractioning by digitonine will be made during the study of cholesterol.

of papaine and that of hot chlorhydric hydrolysis. (Table III)

TABLE III

Results given in gammas for 100 cm^3 of normal human plasma

	Before hydrolysis	After papaine (extract A + B)	After chlorhydr. hydrolysis preceded by papaine digestion (extracts C + D)	After direct chlorhydr. hydrol. (i.e., not preceded by papaine digestion)
	(*)			
17-OH cortic...	a.8	32	4	23
	b.6	27	9	16
	(**)			
17-non-phenol	a.80	280	0	310
cetoster	b.90	195	30	220

(*) Technique Nelson & coll.
(**) Extracts with trichlorethylene at the plasma pH. The Cahen and Salter techniques.

Naturally we only use hot chlorhydric hydrolysis as a research medium and with the necessary reservations for a destructive process. We do not intend to offer it as a method; it is a last resort for discovering relations which are otherwise detectable.

From this first test we conclude that human blood plasma contains: (1) proteinic relations unknown to date with 17-hydroxycorticosteroids; (2) a relatively high quantity of 17-cetosteroids attached to the proteins (on an average, approximately 240 mmg per liter; (3) non-proteinic (?) relations of the same steroids, hydrolyzed by hot concentrated HCl.

Second cause for error:

Imaginary identification of cholesterol in biological products

Our knowledge of cholesterol in blood and in tissue is more often a sort of "tradition" than actual science. Cholesterol was separated in blood and in various tissues by a colored reaction - Liebman's, applied mostly by Grigaut (1910-1913) - at a time when only 4 or 5 steroids were known among which cholesterol was the only one to cause a reaction.

Research conducted by the use of this test rarely culminated in satisfying idnetifications. It was under these circumstances that the work on lipoproteins started between 1925 and 1930. When at this time a product extracted from blood plasma by a solvent of lipids, crystalized, was Lieberman-positive and even formed a digitonide - could there be any doubt that the cases (infrequent) during which some was produced could be anything but cholesterol?

And after all, how many times is cholesterol mentioned in the publications of that era without any mention of proof or method.

These so very frigid convictions of the heroic epoch have often not been verified by subsequent generations and have become "classical" by force of time.

Such we believe to be the history of what we call "the tradition of cholesterol". This tradition will possibly brake the advance of blood and tissular steroid studies for a long time to come.

What must be criticized in the current methods of cholesterol identification in biochemistry or in medical chemistry?

The Lieberman-Burchardt reaction is at the base of most of these methods.

This is often preceded by the formation of a digitonide, as in the case of Schoenheimer and Sperry (67).

The digitonide formation is evidently not specific of cholesterol. Twelve of the thirteen hydroxyle steroids in C_3 suprarenal extracts, are precipitable by digitonine.

Moreover the digitonide formation is neither specific of hydroxyles-B (see (17), Page 60) nor of steroids since phenol, the d- and l- a-terpineols, butanol, amylic alcohol, etc., are precipitated by digitonine (*).

Lieberman-Burchardt's colored reaction is as unspecific as the former. This reaction is positive among others, with all the 3-hydroxy-$\Delta^{5,6}$ sterols of the general type (I) and with all the Δ^{7}-stenols of type (II). It is therefore positive in the animal organism, not only with cholesterol but also for example with Δ^{5}-pregnene-3 (B)-ol-20-one and with dehydroepiandrosterone.

(Fig. (Page 581))

We have thus noted that a mixture of 40 p. 100 (in weight) of cholesterol, of 40 p. 100 Δ^{5}-pregnenolone and 20 p. 100 desoxycorticosterone, following Lieberman's reaction for dosage, corresponds roughly to 100 p. 100 of colored cholesterol equivalent.

The interference with Δ^{5}-pregnenolone is especially serious since this body is present in the steroid secreting glands - and doubtless in the blood - and because there are reasons for believing it to be more of a forerunner of corticosteroids than cholesterol itself (see the works of Hechter, Zaffaroni,

(*) Natigine gives slightly different but also non-specific results.

Pincus, already mentioned).

Let us also note that Δ^5-pregnenolone - like dehydroepiandrosterone - forms a digitonide and that, therefore, the confusion with cholesterol is fatal and complete. Theoretically it is possible not to find a trace of cholesterol where present day methods claim to find great quantities.

This critique calls for 3 conclusions:

1. The usual cholesterol research and dosage methods, based on the formation of digitonide and on the Lieberman reaction (or on any other related reaction, such as Salkowski's) have neither chemical nor physiological value.

2. The colored reactions which have first been studied must be eliminated from all future cholesterol research and a correct identification method must be adopted.

3. From a strictly biological point of view, all the works on cholesterol made exclusively by the methods in question should be reviewed.

Proposed method

1. Extractions and fractionings

One must start with at least 60 cm^3 of blood plasma. One may conduct the extraction according to the technique of Schoenheimer and Sperry, modified by Hawk, Oser and Summerson (67). After filtration, the extract is evaporated until dry. The residue is carried to stage 3 of the technique which we just described.

This last technique may also be completely applied. One finishes with a chromatography on alumina.

2. Tracking down

An aliquot part of every sample is dosaged, after evaporations, with the Lieberman reaction so as to track down fractions susceptible of being cholesterol.

3. Identification

The samples corresponding to each positive Lieberman chromatographic peak are mixed by peaks. The mixture corresponding to a peak is divided into 3 equal parts which are evaporated until dry in damaged recipients. They are weighed.

Supposing that it concerns cholesterol, the three residues are prepared respectively:

a. bibromure, $C_{27}H_{46}OBr_2$, p.f. 116-117° or 123-124°; (a) $_D$-43,5 ($CHCl_3$).

b. the acetate, p.f. 114-115°; (a) $_D$-47,5°($CHCl_3$).

c. bibromure acetate: p.f. 115° and 117° (dimorphism).

The physical constants of these three derivatives allow a strict identification of cholesterol, in keeping with the operating conditions which have just been described.

4. Dosage

It can be done from all three derivatives by gravimetry. The result will be expressed in free cholesterol.

Results

The results will be published in detail after the termination of the research. Following are some partial results.

The use of the Schoenheimer and Sperry (67) method gave in 4 out of 5 cases, a colored cholesterol equivalent in perfect agreement with the strict identification made by our process. The Lieberman colored equivalent

contained approximately 10 p. 100 of a non-identified residue differing from cholesterol in 1 out of 5 cases (experiments always conducted with 60 cm^3 of blood plasma per subject). (Table IV).

TABLE IV

True cholesterol from normal human blood plasma

Subject	Colored Equivalents (Schoenheimer & Sperry)	True Cholesterol (personal process)
1. M. Bert.	195	199
2. M. De Mo.	210	202
3. M. Van Cal.	235	235
4. M. Mol.	260	267
5. M. Ruys.	245	222 + 24 mmg. non-cholesterol residue

We discovered the following results in a non-compensated case of diabetes (after fasting):

glycemy (Nelson): 460 mg p. 100

"cholesterolemy" (Schoenheimer & Sperry): 400 mg p. 100

true cholesterol (personal process): 275 mg p. 100

+ non-cholesterolic residue (being studied): 107 mg p. 100

The true cholesterol was identified, after fractioning and chromatography on aluminum, by bibromure (p.f. mixed with an authentic very pure sample: 124°), by acetate (p.f. mixed 114-115°) and by bibromure acetate (p.f. mixed 115-117°).

In this diabetes case, the classical "cholesterol" was therefore contaminated by approximately 30 p. 100 of Lieberman positive products which differ from cholesterol.

It is probably superfluous to insist on the causes of error of the classical research and dosage methods of cholesterol in biological products. A part of the problem of the "omnipresence" and of the high percentage of cholesterol is explained by the usual nonspecific estimates of this steroid (*).

We have tried to draw attention to a partly virgin field which is partly obscured by prejudice.

We have attempted to paint as complete a picture as possible, while stressing the delicate or uncertain points.

The discussion has shown that it is indispensible to seek more precise methods of analysis and we have proposed two methods which from the first results appear encouraging for cholesterol and for the extraction of steroids connected with proteins.

Our conclusions on cholesterol in biochemistry must have appeared particularly misleading. While the cholesterol question should in fact be partially reconsidered in biochemistry, the problem hardly appears that important in current medicine. Routine procedures give generally satisfying results for the diagnostic in this field.

There is no doubt that the steroidoproteinic relations and the cholesterol problem to which it so closely related, forms a field of experience to which rich results call the investigator.

(*) This work on blood and tissue cholesterol was made possible because of the help of M. Christiaens, of Brussels and by the Roussel firm.

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